

Comparative Two-dimensional Gel Analysis and Microsequencing Identifies Gelsolin as One of the Most Prominent Downregulated Markers of Transformed Human Fibroblast and Epithelial Cells

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Abstract. A systematic comparison of the protein synthesis patterns of cultured normal and transformed human fibroblasts and epithelial cells, using two-dimensional gel protein analysis combined with computerized imaging and data acquisition, identified a 90-kD protein (SSP 5714) as one of the most striking downregulated markers typical of the transformed state. Using the information stored in the comprehensive human cellular protein database, we found this protein strongly expressed in several fetal tissues and one of them, epidermis, served as a source for preparative two-dimensional gel electrophoresis. Par-

tial amino acid sequences were generated from peptides obtained by in situ digestion of the electroblotted protein. These sequences identified the marker protein as gelsolin, a finding that was confirmed by two-dimensional immunoblotting of human MRC-5 fibroblast proteins using specific antibodies and by coelectrophoresis with purified human gelsolin. These results suggest that an important regulatory protein of the microfilament system may play a role in defining the phenotype of transformed human fibroblast and epithelial cells in culture.

TWO-DIMENSIONAL gel electrophoresis has been widely used to quantitatively and qualitatively analyze the protein composition of whole cells (O'Farrell, 1975; Garrels, 1979; Celis et al., 1984) and to systematically compare protein synthesis of cells in response to various stimuli (e.g., Celis et al., 1989, and references therein). Given the complexity of the protein patterns, computerized imaging and data acquisition have been introduced to compare results obtained from various cell types or from cells grown under different conditions, to identify coregulated proteins or proteins specific for certain phenotypes and finally to accumulate information on proteins of interest (Celis et al., 1989; Garrels, 1979, 1989).

Cell transformation has been extensively studied using this approach (Bravo and Celis, 1982; Celis et al., 1989; Garrels and Franza, Jr., 1989, and references therein). Comparison of overall protein expression patterns in pairs of normal and transformed cells have revealed proteins that could be directly or indirectly involved in properties associated with the transformed state, such as derangement of the microfilament system, change in cell shape and motility, loss of control of proliferation, and loss of contact inhibition. Proteins of interest could then be recognized by co-migration with known proteins or by immunocrossreactivity using characterized antibodies. More recently, direct NH₂-terminal or indirect internal microsequence analysis has been used for identification of protein spots recovered from two-dimensional gels (Aebershold et al., 1987; Bauw et al., 1987, 1989).

Here, we have used the comprehensive human cellular protein databases (Celis et al., 1988b, 1989) to search for one of the most prominent and specific protein markers for transformation of cultured fibroblastic and epithelial cells. Using partial amino acid microsequencing, immunoblotting, and co-migration electrophoresis, we identified gelsolin as one of the most striking downregulated marker proteins. This observation is discussed in terms of microfilament organization in normal and transformed cells.

Materials and Methods

Cultured Cells and Tissues

Normal and transformed human cells were grown as monolayer cultures in DME containing 10% (vol/vol) FCS and antibiotics (penicillin at 100 U/ml and streptomycin at 50 µg/ml). Fetal human skin dissected from a 4-mo normal human male fetus was used as a source of protein for microsequencing. These experiments have been approved by the Ethical Scientific Committee of the Aarhus Amtskommune.

Labeling of Cells with [³⁵S]Methionine

Cells grown in microtiter wells (Nunc, Roskilde, Denmark) were labeled for 14 h with 0.1 ml of laboratory-made DME (1 g/liter, NaHCO₃) lacking methionine and containing 10% dialyzed FCS and 100 µCi of [³⁵S]methionine (SJ204; Amersham Corp., Arlington Heights, IL) (Bravo et al., 1982). The procedures for running two-dimensional gels, Coomassie blue and silver staining, and two-dimensional immunoblotting have been described in detail elsewhere (Bravo et al., 1982; Celis et al., 1988a). Protein synthesis rates were quantitated by counting the radiolabel of each spot by liquid scintillation.

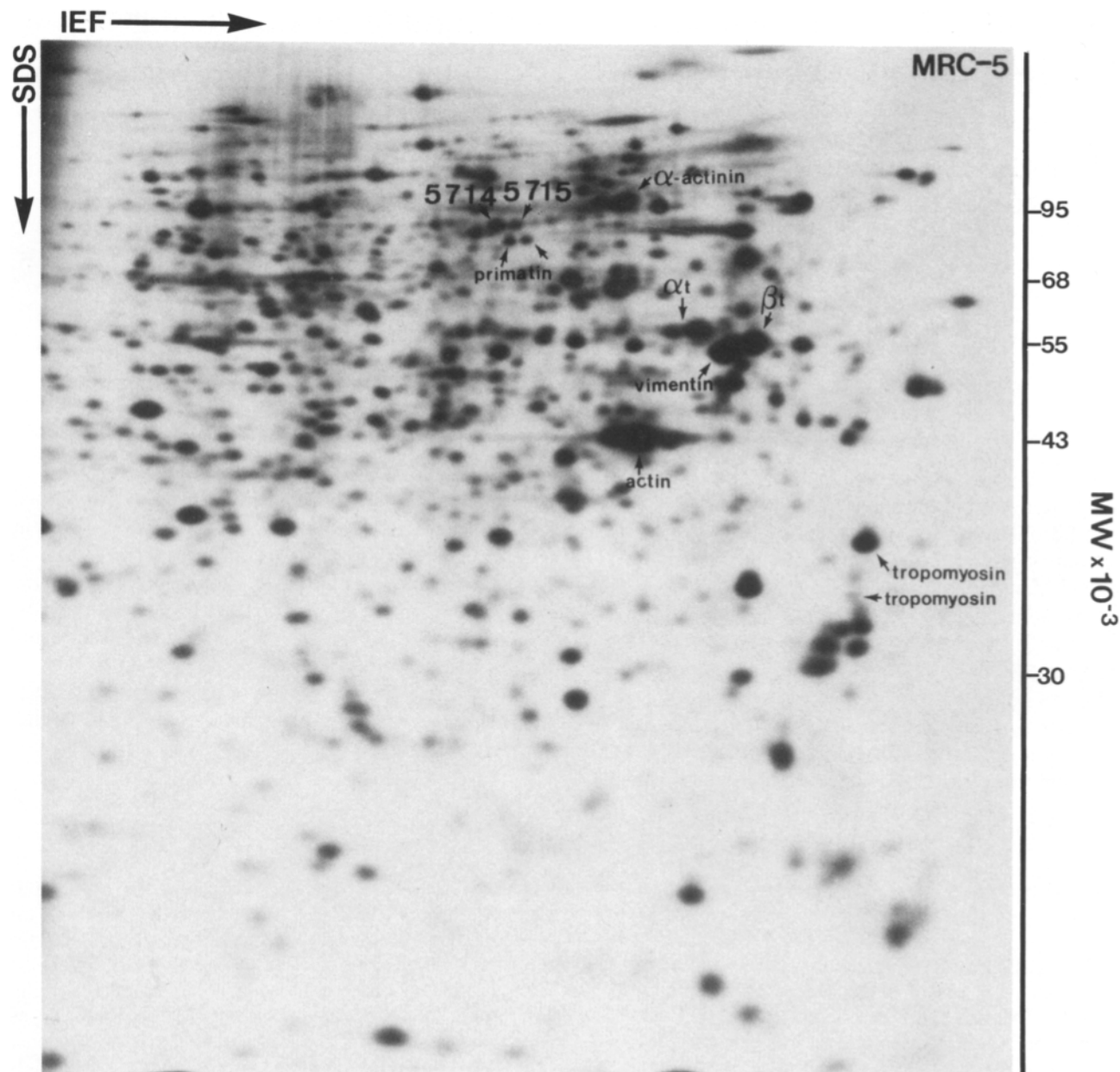


Figure 1. Two-dimensional gel fluorogram (IEF) of ^{35}S -methionine-labeled proteins from MRC-5 human embryonal lung fibroblasts. Proteins were separated horizontally by IEF and vertically by SDS-PAGE (molecular weights of standard proteins are given at the right edge of the picture). SSP 5714 5715 correspond to the transformation sensitive proteins (see also Fig. 2) discussed in this paper. Major cytoskeletal proteins whose rate of synthesis is compared relative to that of SSP 5714 (Table I) are also indicated in this figure. α , α -tubulin; β , β -tubulin. For orientation, the position of primatin is also shown.

Microsequencing

Extracts of fetal human skin tissue were separated by two-dimensional gel electrophoresis and were stained with Coomassie brilliant blue. An aliquot of the [^{35}S]methionine-labeled extract of human MRC-5 fibroblasts was added as a reference to match the Coomassie-stained spots relative to the [^{35}S]methionine-labeled spots. Partial amino acid sequence information was obtained as described by Bauw et al. (1989).

Briefly, the protein spot was collected from 20 Coomassie-stained dried gels. Gel pieces were collected in an Eppendorf tube, and immersed in rehydrating buffer containing 0.1% SDS. After 2 h, the swollen gel pieces were transferred with tweezers into the gel slot of a freshly made gel and overlaid with Laemmli sample buffer (Laemmli, 1970). This new gel contained a stacking gel extending at least 2 cm below the bottom of the slot to assure proper stacking of the protein. The separating gel contained 10%

polyacrylamide. The protein was then electrotransferred from the gel onto PVDF membranes as described by Bauw et al. (1987) and stained with Amido black. The band was excised and subjected to in situ tryptic digestion. Peptides eluting from the membrane in the digestion mixture were separated by HPLC on a C4 (0.46 \times 25-cm) reversed-phase column (Vydac, Separations Group, Hesperia, CA) using a Waters Associates (Milford, MA) system (two pumps, model 510, and a lambda max 810 detector). The column was equilibrated in 0.1% trifluoroacetic acid and a linear gradient from 0 to 70% acetonitrile in 0.1% trifluoroacetic acid was applied over 70 min. The gradient was started 10 min after sample injection. Eluting peptides were recorded by adsorption at 214 nm, collected by hand in Eppendorf tubes (made by Brinkmann Instruments Co., Westbury, NY) and dried in a Speed Vac concentrator (Savant Instruments, Hicksville, NY).

Peptides were selected for amino acid sequence analysis on the basis of peak height and resolution. The sequence was determined with a sequenator

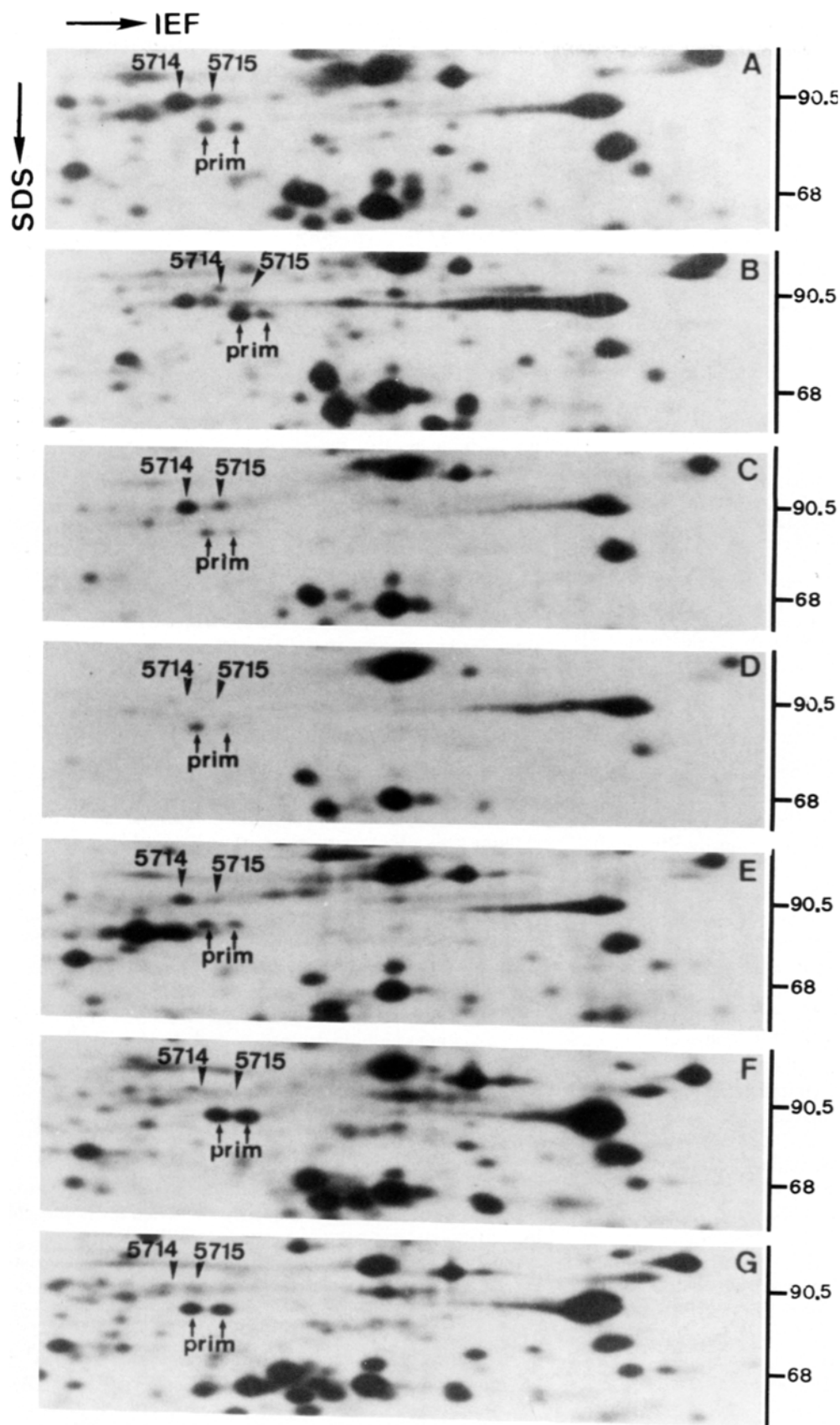


Figure 2. ^{35}S -methionine-labeled proteins in pairs of transformed and untransformed cells. MRC-5 (A) and SV40-transformed MRC-5 (B); WI38 (C) and WI38 SV40 (D); keratinocytes (E) and SV40-keratinocytes (F). G shows the AMA epithelial cell pattern. Only a fraction of the fluorogram is shown covering the region of SSP 5714 and SSP 5715 and primatin. The gel orientation is as in Fig. 1.

Table I. Quantitation of Gelsolin (SSP 5,714) Relative to the Major Cytoskeletal Proteins in Normal and SV40-transformed MRC-5 Fibroblasts (MRC-5 V2)

Protein	MRC-5	MRC-5 V2
Gelsolin (5,714)	363	39
Actin ($\beta + \gamma$) (7,423)	18,838	10,010
β -Tubulin (8,517)	3,023	3,228
α -Actinin (6,716)	1,237	872
Tropomyosin (9,215)	697	315
Tropomyosin (9,226)	45	76

The rate of synthesis was measured by liquid scintillation counting of the different spots. Values given (in counts per minute) are the average of the five different measurements. The same number of counts were applied to each gel. Numbers between brackets refer to the protein numbering in the amnion protein data base (Celis et al., 1989).

(model No. 470A; Applied Biosystems Inc., Foster City, CA) equipped with an on-line phenylthiohydantoin amino acid analyzer (model No. 120A). The instrument was operated according to the instruction of the manufacturer. Obtained sequences were compared with sequences present in the Protein Identification Resources NBRF databank and the SWISS protein sequence bank.

Gelsolin was purified from human serum using the method of Bryan (1988) and obtained in a homogenous form by an additional fast-protein liquid chromatography step. Immunoblotting was carried out with antibodies specific for human gelsolin kindly provided by Dr. H. Yin (Hematology-Oncology Unit, Harvard Medical School).

Results

Fig. 1 shows the [35 S]methionine-labeled protein two-dimensional gel pattern (IEF) of normal human MRC-5 fibroblasts. For orientation, we have indicated the transformation sensitive protein relative to the position of easily identifiable marker proteins such as tropomyosins, total actin, vimentin, α - and β -tubulin, primatin, and α -actinin (Celis et al., 1989). This protein consists of at least two coregulated variants that correspond to SSP 5714 and 5715; (90.5) in the MRC-5 protein database (Celis et al., 1989). Visual comparison of fluorograms from normal human MRC-5 fibroblasts (Fig. 2 A) and their SV40-transformed counterparts (MRC-5 V2) revealed a drastic decrease in SSP 5714 and 5715 associated with the transformed state (Fig. 2 B). Further counting of the [35 S]methionine label contained in the SSP 5714 spot relative to that of total radiolabel applied on the gel revealed a ninefold decrease (Table I). Other proteins like total actin showed a moderate decrease while β -tubulin remained constant. Also the level of tropomyosin SSP 9215 was decreased, while that of tropomyosin SSP 9266 with an apparent low molecular weight showed a considerable increase (see also Celis et al., 1989). Consultation of the annotation databases (Celis et al., 1988b, 1989) revealed that SSP 5714 and 5715 are consistently downregulated. This is further illustrated in Fig. 2 where the two-dimensional protein expression patterns of other couples of transformed and untransformed cells are compared: WI38 versus WI38-SV40 and keratinocytes versus SV40-keratinocytes (K14). Further analysis of the AMA cell protein pattern (Fig. 2 G) and that of HeLa, A431, WISH, and Fl-amnion cells (results not shown) adds further support to the conclusion that SSP 5714 is a general downregulated protein marker typical of the transformed state of cultured fibroblastic and epithelial cells. The

Table II. Quantitation of the Decrease in Gelsolin Synthesis in Transformed Cells Relative to Actin

Ratio actin/gelsolin (5,714)	
MRC-5	52
WI38	48
keratinocytes	110
MRC-5 SV40	257
WI38 SV40	359
SV40 keratinocytes	> 300
AMA cells	> 300

extent of downregulation is given in Table II, where ratios of radiolabel in total actin (the sum of β - and γ -actin) versus that in SSP 5714 are listed for the cell types shown in Fig. 2. The synthesis of the transformation-sensitive marker relative to that of actin is decreased by a factor 5 and 7 in SV40-transformed MRC-5 and WI38 cells, respectively, and at least by a factor 3 in K14 cells. The latter is difficult to measure as normal keratinocytes already express low levels of SSP 5714.

To prove that the observed variations in 35 S-label were related to similar changes in protein content, we performed a silver-staining on the MRC-5 and MRC-5 SV40 protein gels (Fig. 3). The changes in protein patterns were similar in the silver-stained gels as in the fluorograms. This excludes the possibility that the observed decrease in 35 S-label was because of a decrease in protein turnover or degradation in the transformed cells.

Substantial levels of the SSP 5714 have been observed in primary and secondary human fetal fibroblasts from ear, skin, kidney, and meninges as well as in normal human epithelial amnion cells. *Xeroderma pigmentosum* fibroblasts (XP3Br, XP25RO) also synthesize abundant levels of this protein. So far, all fetal human tissues analyzed exhibit high levels of this protein: these include heart, tongue, stomach, ureter, submaxillary glands, pectoral muscle, hypophysis, eye, brain, pancreas, and large intestine (for illustration see Fig. 4).

To perform microsequencing, fetal human skin was selected as a source of the protein. Several SSP 5714 spots cut out of Coomassie blue-stained two-dimensional gels were processed as described in Materials and Methods. Three peptides were selected from the HPLC chromatogram for sequence analysis and the following results were obtained: peptide 1, XQPVQVAEGSEPDGFXE; peptide 2, XXQGFE-XAXFLG; and peptide 3, QGQIYNXQGAQSXQ, with X being an unidentifiable residue either due to contamination or to weak signals. These sequences matched those present in human gelsolin: peptide 1, residues 600–616; peptide 2, residues 121–132; and peptide 3, residues 459–473 (Kwiatkowski et al., 1986). Identical results were also obtained for spot SSP 5715 (results not shown). The identity between SSP 5714, 5715, and the cytosolic gelsolin variants was further confirmed by two-dimensional immunoblotting of MRC-5 proteins using specific gelsolin antibodies (Fig. 5). Finally, we confirmed the above results by a comigration experiment with purified gelsolins and an 35 S-labeled extract from normal human MRC-5 fibroblasts. The gelsolin was purified from a batch of pooled sera that were contaminated by tissue-extracts and therefore contained both the cytosolic and secreted variants. The identity of these variants was verified by

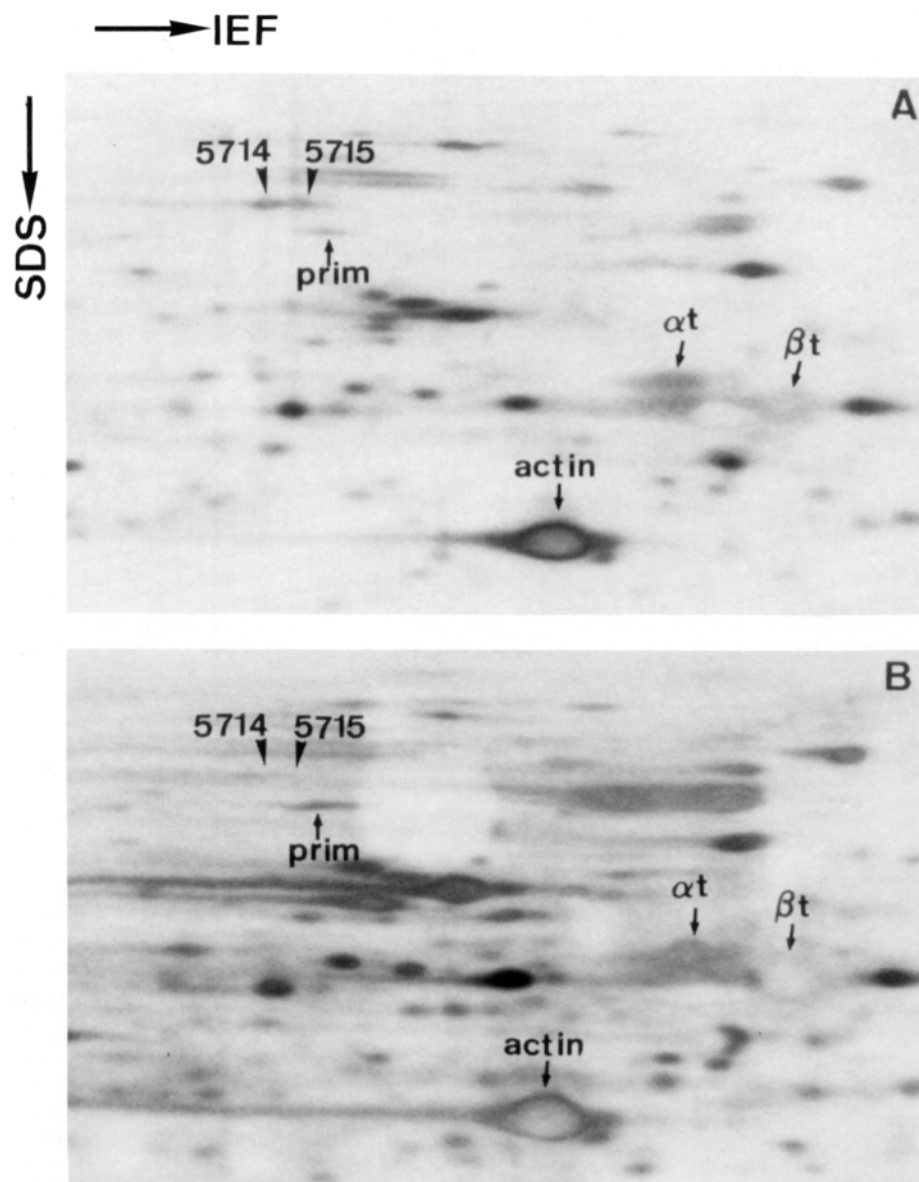


Figure 3. Silver-stained two-dimensional protein patterns of the area containing the transformation-sensitive marker in extracts of MRC-5 (A) and MRC-5 SV40 (B) cells. Notice in the transformed cell the dramatic decrease of amounts of SSP 5714 relative to that of other proteins such as α and β tubulin (α_t and β_t), actin, and primatin (*prim*). The amount of total protein loaded on A was half that of B.

NH₂-terminal sequence analysis on each of the forms separated by 1D-polyacrylamide gel electrophoresis (Yin et al., 1984). Only the cytosolic variants showed co-migration with the identified transformation-sensitive marker from ³⁵S-labeled MRC-5 fibroblast extracts (data not shown).

Discussion

Using a systematic analysis of two-dimensional gel protein patterns in pairs of normal and transformed cultured human fibroblast and epithelial cells (MRC-5 and MRC-5 V2, WI38 and WI38-SV40, keratinocytes and SV40 keratinocytes), in transformed cell-lines of human epithelial origin (AMA, HeLa, A431, WISH, Fl-amnion), in primary cell cultures, and in various normal human cells and tissues, we have identified a specific marker protein that is substantially down-regulated in the "transformed state." This 90-kD protein, which corresponds to SSP 5714 and 5715 in the AMA data base (Celis et al., 1989), was identified by protein-chemical, physicochemical and immunochemical criteria as gelsolin.

Gelsolin is encoded by a single gene but expressed, due to alternative processing, in a cytoplasmic and a secreted form (Kwiatkowski et al., 1986). Both forms are synthesized by a variety of cell types including fibroblasts and epithelial cells (Kwiatkowski et al., 1988). They display a slightly different molecular mass, due to an NH₂-terminal extension selectively added to the secreted form and each of these forms separates in different isoelectric variants (Chaponnier et al., 1985). Only cytosolic gelsolin is considered here, and it is not known whether the secreted form is regulated in a similar manner. As the level of secreted gelsolin in fibroblasts and epithelial cells is low and difficult to accurately measure in a systematic way, we have been unable to address this interesting issue. In this respect, it should be mentioned that Kwiatkowski et al. (1988) was able to demonstrate that the two forms of gelsolin are independently regulated in normal tissues.

Gelsolin is one of the most abundant actin regulatory proteins of nonmuscle cells (e.g., Yin, 1987), and the finding of its downregulation may help us to understand the derange-

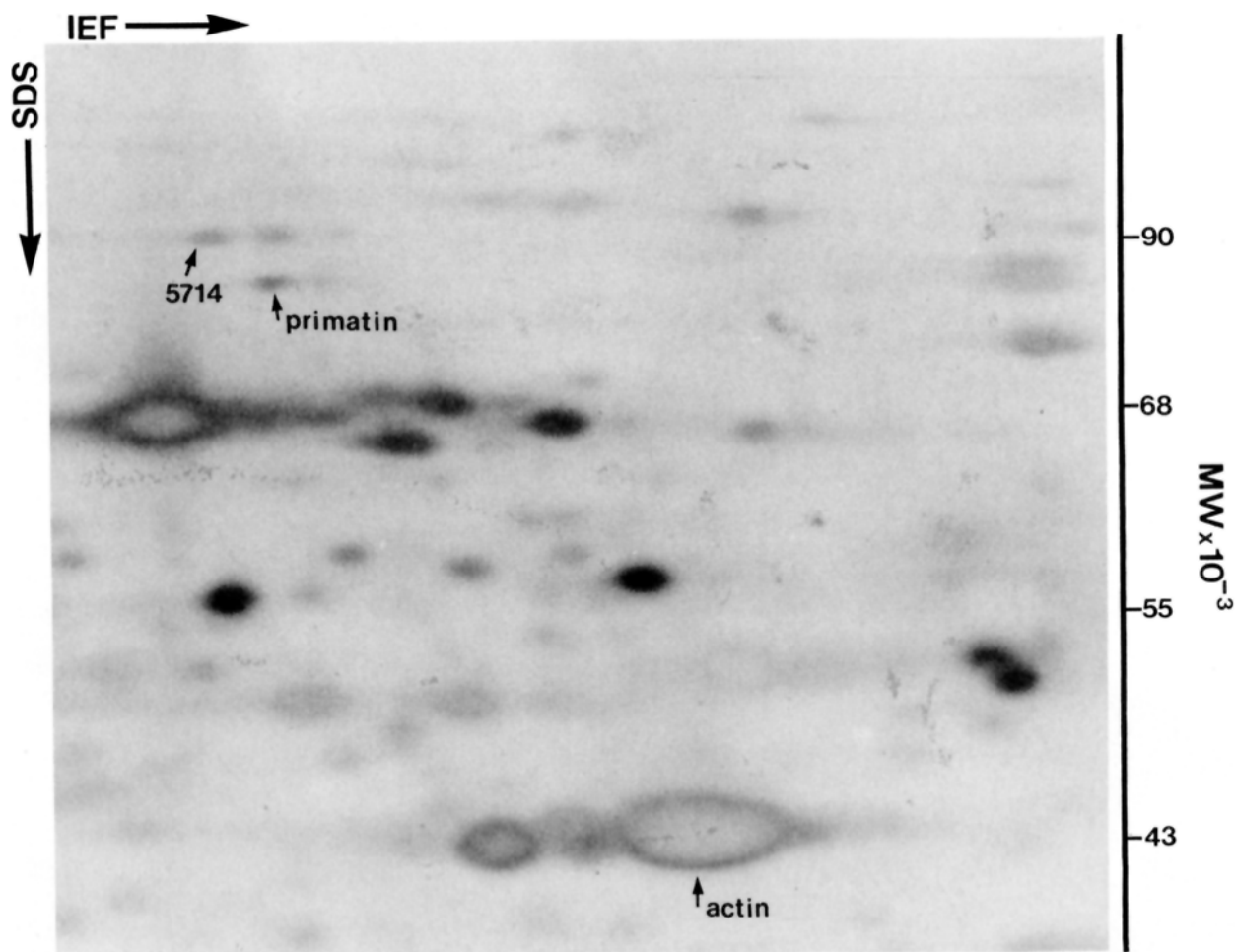


Figure 4. Silver-stained two-dimensional protein pattern of fetal human large intestine. Only a fraction of the gel is shown. The orientation of the gel is as in Fig. 1.

ment of actin structures associated with the acquisition of the transformed phenotype (e.g., Pollack et al., 1975; Veredame et al., 1981; Boshek et al., 1981). A mechanism by which gelsolin could control assembly in the cortical cytoplasm in response to two intracellular signaling systems (Ca^{2+} and polyphosphoinositides) has been proposed recently (Hartwig and Yin, 1988). Furthermore, it has been suggested that this protein is functionally linked to receptors for chemotactic peptides and other external signals (Chaponnier et al., 1987). A significant reduction of this pivotal protein could thus directly account for the disruption of the cortical actin network and loss of motility and chemotaxis observed upon cell transformation.

The situation is likely to be more complex as it is known that cell transformation is associated with (less marked) downregulation of other microfilament or microfilament-associated proteins. This includes α smooth muscle actin (Leavitt et al., 1985; Garrels and Franza, 1989), the higher molecular weight tropomyosins (Hendricks and Weintraub, 1981; Matsumura et al., 1983; Leavitt et al., 1986; Celis et al., 1989; and this study), nonmuscle caldesmon (Koji-Owada et al., 1984), profilin (Kwiatkowski, 1988), smooth muscle myosin light chain 2 (Kumar et al., 1989), protein C4 (Shapland et al., 1988), and actin-binding protein

(Kwiatkowski, 1988). Some of these proteins may act synergistically to organize and to control actin organization. For instance, profilin is thought to dissociate from actin at high phosphoinositol-biphosphate and low Ca^{2+} , thus providing polymerization competent actin monomers after gelsolin has been released from the newly formed actin nuclei (Hartwig and Yin, 1988). High molecular weight tropomyosins display a protective effect on actin filaments for gelsolin, and this effect is even increased by nonmuscle caldesmon (Ishikawa et al., 1989). Actin-binding protein is involved in the formation of an actin filament network in the cell cortex (Hartwig and Yin, 1988). Thus, the reason underlying changes in the microfilament organization upon transformation may be a coordinated downregulation of all the elements of a structural and functional actin organization unit, rather than the effect of a single protein on the system. Some of these proteins are regulated at the transcriptional level (Kwiatkowski, 1988), but their decrease may also (in part) result from a loss of organization, by which they become more prone to degradation.

Although, to our knowledge, this is the first report where downregulation of gelsolin has been systematically noticed in a large number of cultured transformed cell-lines, observations in the same line as those reported here, have been

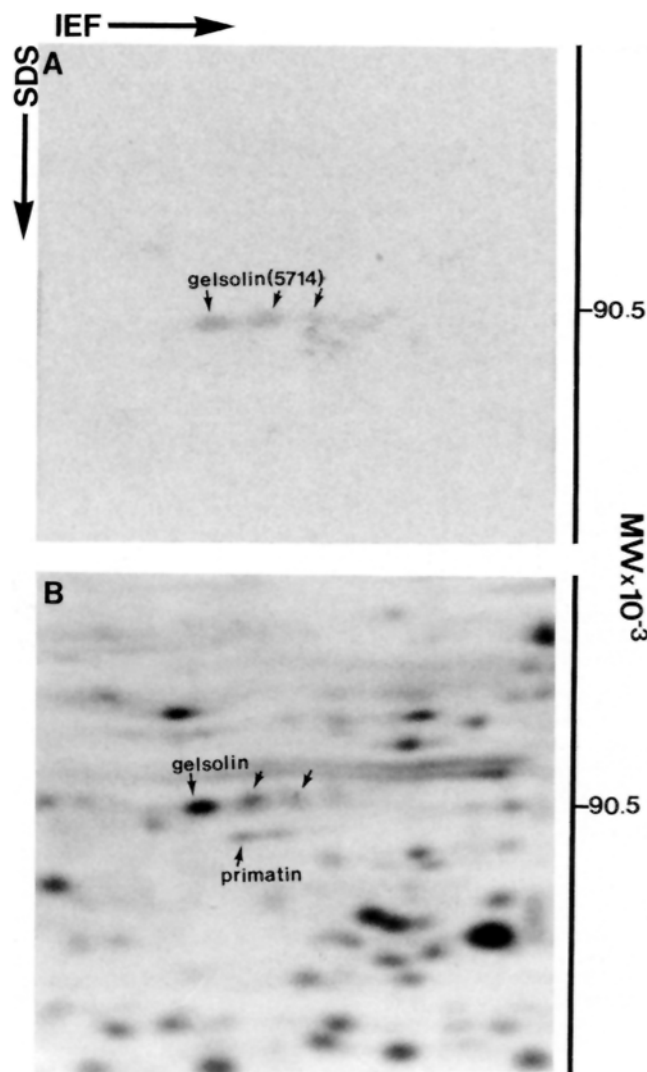


Figure 5. Identification of gelsolin in MRC-5 fibroblasts by immunoblotting. (A) the blot; (B) the corresponding autoradiogram. Only a fraction of the gel is shown. The gel orientation is as in Fig. 1. The cytosolic gelsolin variants are indicated. Their position relative to primatin is shown.

made previously. They were derived by directly analyzing separate cell-lines individually by immuno- and Northern blotting. Human myeloid cells (HL60 and U937) that have lost the potential for growth and division after tetradecanoylphorbolacetate treatment show increased synthesis and levels of this protein (Kwiatkowski, 1988). Similarly, differentiation of the murine embryonal carcinoma cell-line PC13 was accompanied by the drastic increase of gelsolin (Dieffenbach et al., 1989). These analyses together with the studies described in this report were carried out on cultured cell systems, which are homogeneous and easily amenable for quantitative analysis. However, it is likely that the gelsolin downregulation also applies when normal and tumor tissues are compared. Indeed our analysis made on a large variety of different tissues consistently revealed a relatively high expression of this protein. In the case where tumor tissue was analyzed for gelsolin expression, the protein was significantly downregulated. Thus, using an immunocytochemical

approach, Chaponnier and Gabbiani (1989) showed that in human breast carcinoma epithelial cells gelsolin levels were consistently decreased. Taken together, these results suggest that low levels of gelsolin are typical of cells with an undifferentiated and proliferative phenotype, while high gelsolin expression seems to be consistently observed in differentiated and nonproliferative cells. Gelsolin may therefore not only be important in the organization of the cortical network, chemotaxis and motility, but may also play a role in cell division (e.g., through the formation of the contractile ring). This idea is further supported by observations that cells grown in the presence of cytochalasins, a group of fungal metabolites shown to bind to the barbed end of the actin-filaments in a similar manner as gelsolin (Flanagan and Lin, 1980), are blocked in telophase and enucleate (Fenech and Morley, 1986; Godman and Miranda, 1978). From these data, it seems that gelsolin downregulation could be a requirement to assure normal cell division. As gelsolin is regulated by two second messengers (Ca^{2+} and polyphosphoinositides) with opposing effects, it is possible that the only way to stop any gelsolin activity during cytokinesis is by eliminating the protein from the cell. This hypothesis could be verified by studying the rate of gelsolin synthesis and accumulation throughout the cell cycle. Furthermore, transfection experiments in which gelsolin is overexpressed (using a constitutive promoter) or downregulated (by using antisense RNA expression), may reveal new phenotypes relevant to gelsolin's function.

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